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HNF1α controls renal glucose reabsorption in mouse and man

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Recently it has been shown that dominant mutations in the human hepatocyte nuclear factor 1 α (HNF1α) gene, encoding for a homeoprotein that is expressed in liver, kidney, pancreas and intestine, result in maturity onset diabetes of the young type 3 (MODY3). HNF1α-null mice are diabetic, but at the same time suffer from a renal Fanconi syndrome characterized by urinary glucose loss. Here we show that MODY3 patients are also characterized by a reduced tubular reabsorption of glucose. The renal murine defect is due to reduced expression of the low affinity/high capacity glucose cotransporter (SGLT2). Our results show that HNF1α directly controls SGLT2 gene expression. Together these data indicate that HNF1α plays a key role in glucose homeostasis in mammals.

INTRODUCTION

Hepatocyte nuclear factor 1 α (HNF1α) is a transcriptional activator that was initially shown to be involved in the regulation of a large set of hepatic genes including albumin, α- and β-fibrinogen and α-1 antitrypsin (Cereghini *et al.*, 1987; Courtois *et al.*, 1987; Frain *et al.*, 1989; Baumhueter *et al.*, 1990; Chouard *et al.*, 1990). HNF1α is a dimeric homeodomain protein that is expressed in liver, kidney, pancreas and the digestive tract (Ott *et al.*, 1991). The modular structure of the protein includes a dimerization domain encoded by the first 32 amino acids, a variant DNA-binding homeodomain and a transactivation domain in its C-terminal part. We have shown that HNF1α-deficient animals are normally born and that a fraction of them die postnatally around the weaning period. Mutant animals are wasted and suffer from hyperphenylalaninemia caused by the absence of hepatic phenylalanine hydroxylase transcription (PAH) (Pontoglio *et al.*, 1996, 1997). Mutant animals also suffer from a renal Fanconi syndrome characterized by severe glucose, phosphate and amino acid urinary wasting (Pontoglio *et al.*, 1996).

Recent investigations have revealed a clear association between autosomal dominant mutations in the human HNF1α gene and a particular form of type 2 diabetes called maturity onset diabetes of the young type 3 (MODY3) (Yamagata *et al.*, 1996). These patients suffer from an insulin secretion defect that appears frequently before the age of 25. A link between mild diabetes and primary renal glucosuria has been reported (Ackerman *et al.*, 1958; Tattersall, 1974) and a defect in renal glucose reabsorption in one MODY3 family has been described (Menzel *et al.*, 1998). However, a cause–effect relationship between MODY3 mutations and the renal defect has not been clearly demonstrated. While *HNF1α*$^{+/-}$ heterozygous mice are healthy, *HNF1α*$^{-/-}$ homozygous animals present with a clear defect in insulin secretion that parallels that of MODY3 patients (Pontoglio *et al.*, 1998). The drastic insulin secretion defect observed in the HNF1α-deficient animals does not result in a strong hyperglycemia, possibly because of the renal urinary glucose wasting.

The molecular mechanisms at the basis of the renal Fanconi syndrome are not fully understood. Glucose is reabsorbed through a secondary active transport that is accounted for by a two-step process. The first stage is an 'uphill' transport across the luminal brush-border membrane of the proximal tubular cells. In this first step, the metabolite is taken up by specific sodium-

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dependent cotransporters localized on the luminal brush-border membrane of tubular cells. These cotransporters bind glucose and sodium at the same time and transport them inside the cell. The energy necessary for this process is provided by the electrochemical gradient of sodium. The second stage is a downhill transport across the basolateral membrane by a passive facilitated diffusion mediated by a different family of specific carriers. Several types of sodium-dependent cotransporters specific for glucose (SGLT1, SGLT2, SAAT1) (Hediger *et al.*, 1987; Kanai *et al.*, 1994; Mackenzie *et al.*, 1994) have been characterized.

We describe here the identification of the gene responsible for the renal glucose reabsorption defect observed in the *HNF1α*$^{-/-}$ mice and demonstrate that it is controlled by HNF1α. In addition we show that renal proximal tubular reabsorption of glucose is reduced in MODY3 patients.

RESULTS

Metabolite uptake in isolated renal proximal tubules

The nature of the defects that lead to the renal proximal tubular dysfunction in HNF1α-deficient mice was unknown. First, we verified whether glucosuria was actually due to a renal autonomous reabsorption defect. To this end, we checked whether renal proximal tubules isolated from wild-type and HNF1α-deficient animals could efficiently take up glucose *in vitro*. α-methyl-glucopyranoside (MGP), a non-metabolized glucose analogue that enters the cell preferentially through the sodium-dependent glucose cotransporters, is taken up much less efficiently in mutant-derived tubules (3.0 ± 0.4 pmol/mg protein) if compared with the controls (7.6 ± 0.6 pmol/mg protein; mean ± SE; $p < 0.005$, $n = 3$) (Figure 1A). Moreover, if we subtract from the total MGP uptake the fraction due to non-specific entry of the substrate that is not inhibited by phlorizin, a specific inhibitor of the sodium-dependent glucose transport, the relative uptake of HNF1α-deficient tubules becomes <25% with respect to the control animals. Conversely, the uptake of 2-deoxy-glucose (2DG), a glucose analogue that is normally taken up only via the basolateral facilitated transport mediated by GLUT2, is similar in mutant and wild-type animals (Figure 1A). Phloretin, a specific inhibitor of the basolateral glucose-facilitated diffusion, equally inhibited the transport in mutant and wild-type mice. In order to characterize better the nature of the defect we also measured the uptake of glucose and galactose on brush-border membranes. Our results indicate that the apical transport of glucose is severely affected (Figure 1B). The transport of galactose, a hexose that can be taken up by SGLT1 but not by SGLT2 (Kanai *et al.*, 1994) nor by SAAT1 (Mackenzie *et al.*, 1994), is normal, indicating that the defect could possibly involve SGLT2 or SAAT1 but not SGLT1. As a control, we also monitored the uptake of alanine, an amino acid that is almost normally reabsorbed in homozygous mutant animals. Our results show that the uptake of this amino acid was not significantly different between HNF1α$^{+/+}$ and HNF1α$^{-/-}$ tubules (Figure 1C).

The secondary active transport of glucose relies on the electrochemical gradient of sodium. However, our results indicated that both Na^+/K^+-ATPase pump activity and ATP concentration

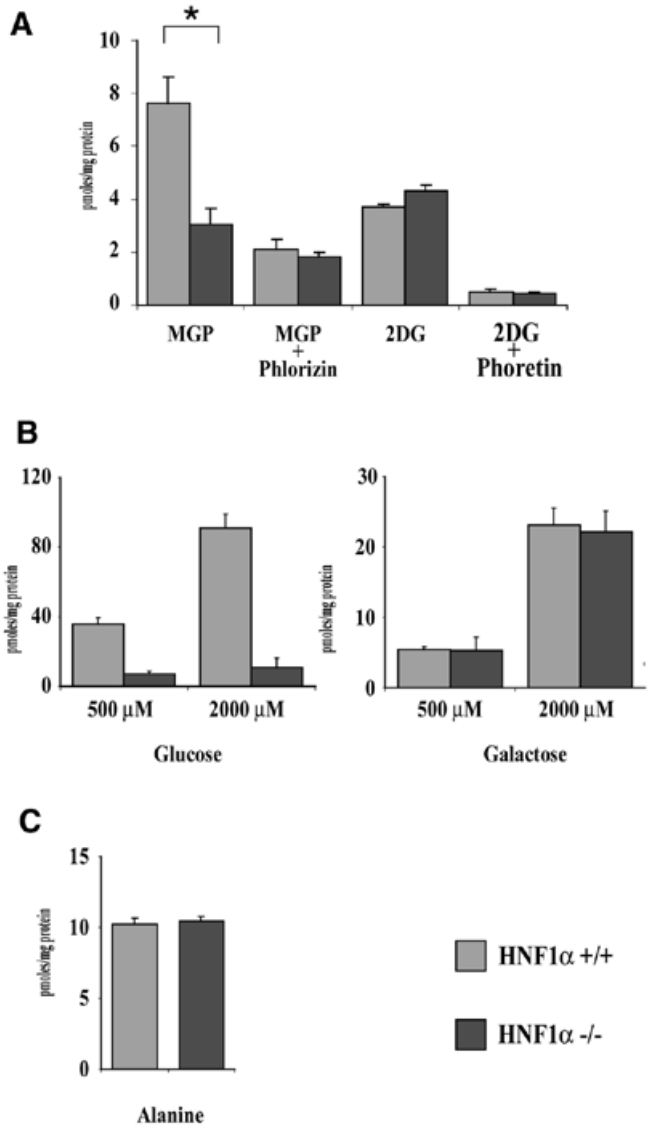

Fig. 1. Renal tubule uptakes. Uptake of glucose or analogues and alanine in renal tubules (**A** and **C**) or in brush-border membranes (**B**) from wild-type (grey bars) and homozygous HNF1α$^{-/-}$ mice (solid bars). α-methyl-D-glucopyranoside, MGP; 2-deoxy-glucose, 2-DG. The results are the mean from three independent experiments. Error bars represent SE. (*) $p < 0.005$.

in proximal tubules were not significantly different between mutant and wild-type mice (data not shown).

The expression of genes coding for specific sodium-dependent cotransporters

In order to unravel the molecular basis of the defective transport, we monitored the expression levels of several renal cotransporters that are believed to be involved in the proximal tubular reabsorption. Northern blot analysis demonstrated that one specific cotransporter was affected by the HNF1α inactivation (Figure 2). In HNF1α-deficient animals, SGLT2, the sodium-dependent glucose cotransporter 2, was expressed at ~10–20%

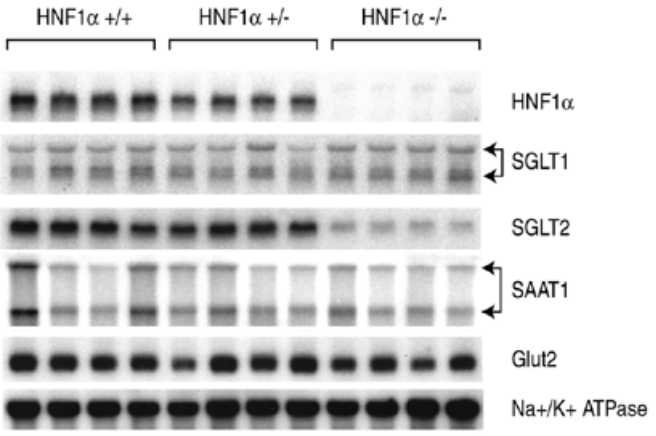

Fig. 2. Northern blot on total kidney RNA. Northern blot analysis on total RNA from kidneys of animals of 12–15 days of age. Four samples for each genotype are shown, from left to right: wild-type, heterozygous and mutant homozygous animals. SGLT1, sodium glucose transporter type 1; SGLT2, sodium glucose transporter type 2; SAAT1, sodium-dependent glucose cotransporter type 2 also known as SGLT3; Glut2, glucose transporter type 2; Na^+/K^+ ATPase α subunit of the Na^+/K^+ ATPase pump.

of the levels observed in heterozygous and wild-type controls. No difference was observed between heterozygous and wild-type animals. SAAT1 and SGLT1, two closely related sodium-dependent glucose cotransporters, were normally expressed. GLUT2, the basolateral sodium-independent glucose carrier, as well as the D2/NAAT amino acid transporter and the α-subunit of the Na^+/K^+ ATPase, were expressed at equivalent levels in all three genotypes.

SGLT2 transcription depends on HNF1α

In order to confirm that HNF1α had a direct effect on SGLT2 gene transcription, we studied the transcriptional control regions of this gene. A λDASH mouse genomic library was screened with a probe spanning the first 500 bp of the rat SGLT2 cDNA (DDBJ/EMBLGenBank accession No. U29881). Four independent overlapping clones were isolated, subcloned and restriction mapped. A 3000 bp fragment, encompassing the putative transcriptional start site deduced from the rat cDNA sequence, was sequenced (DDBJ/EMBLGenBank accession No. AJ292928). It contained 2000 bp of promoter and the first 3 exons (Figure 3A). The computer sequence analysis of this DNA fragment revealed the presence of at least two HNF1 binding sites at positions –45 and –1876. The score of the sequence homology suggested that the proximal site had a higher affinity than the distal. Indeed, our results showed that both sites were bound by a bacterially expressed N-terminal HNF1α moiety (HNT) (Figure 3B). The proximal site was rather efficiently shifted by HNF1α contained in hepatic nuclear extracts, whereas the distal site gave only a weak signal. Short (400 bp) or long (2000 bp) promoter fragments were cloned upstream of a luciferase reporter cassette and cotransfected together with an HNF1α expression vector (RSV–HNF1) (Chouard *et al.*, 1990) in C33 non-hepatic cells lacking HNF1α (Figure 3C). Both constructions were activated by HNF1α in transient cotransfection experiments, indicating that HNF1α plays a direct role in the transcriptional activation of the SGLT2 gene.

MODY3 patients and renal tubular reabsorption

As mentioned above, MODY3 patients are characterized by a defect in insulin secretion. Mice lacking HNF1α have a very similar β-cell pancreatic dysfunction. However, *HNF1α*$^{-/-}$ homozygous mice also suffer from a defect in the renal proximal tubular reabsorption of several metabolites and particularly glucose. To test whether the resemblance between HNF1α-deficient mice and MODY3 patients could also concern the renal phenotype, we monitored the renal function of MODY3 patients. For this purpose, we compared the maximal glucose transport capacity (Tm) of eight MODY3 patients belonging to four families, each affected by different mutations, with a group of six patients with classical late-onset type 2 diabetes mellitus. Our results show that MODY3 patients present with a consistent decrease in glucose reabsorption (Figure 4; Table I). These findings were also strengthened by the presence of fasting glucosuria in the MODY3 patients M3#1, M3#3, M3#4, M3#6 and M3#7 on the day of the investigations, when their fasting serum glucose concentrations were within the normal range. The MODY3 patient M3#5 was also glucosuric but her fasting serum glucose concentration was >10 mM.

DISCUSSION

The present study unravels the molecular basis of the defect in renal glucose reabsorption that was observed in HNF1α-deficient mice. We show that the renal proximal reabsorption failure is due to a kidney-autonomous defect since we have detected a reduced uptake of glucose in isolated tubules *in vitro*. This defect seems to be caused by a drastic reduction in the transcription of a specific glucose sodium-dependent cotransporter (SGLT2).

The reabsorption of glucose in the kidney is carried out by two systems: a high capacity/low affinity and a low capacity/high affinity system (Turner and Moran, 1982). The former seems to be responsible for the uptake of the vast majority of metabolite, especially in the first tract of the tubule when the concentration of glucose is still high. The latter system seems to operate in the more distal part of the proximal tubules, where the concentration of glucose has fallen to low levels. Each system is encoded by a distinct class of cotransporters. It has been suggested that both SGLT2 and SAAT1 are involved in the low affinity/high capacity glucose transport (Kanai *et al.*, 1994; Mackenzie *et al.*, 1994; You *et al.*, 1995), whereas SGLT1 controls the high affinity/low capacity reabsorption (Hediger *et al.*, 1987). In the kidney of *HNF1α* mutant animals, only SGLT2 is affected, whereas SGLT1 and SAAT1 are expressed at normal levels. The fact that proximal tubules from HNF1α-deficient mice can transport galactose with the same efficiency as the control animals further supports the idea that SGLT1 function is not affected in HNF1α-deficient mice. SGLT2 is mainly expressed in the S1 portion of proximal tubules (Kanai *et al.*, 1994). The observed reduction of SGLT2 expression correlates well with previous observations concerning a significantly reduced number of phlorizin binding sites on brush-border membrane vesicles of mutant animals (Pontoglio *et al.*, 1996). This observation

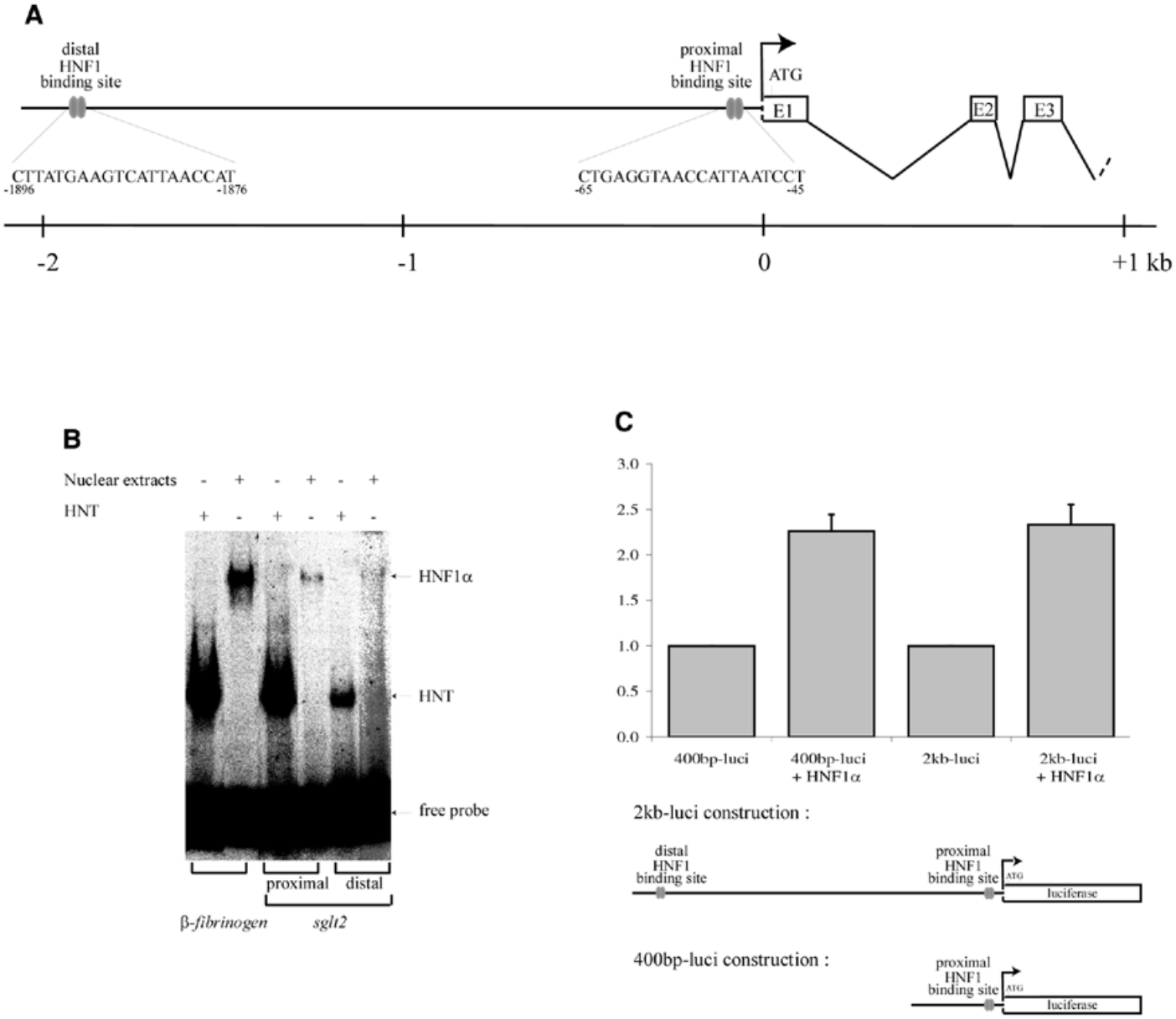

Fig. 3. SGLT2 promoter analysis. (**A**) Schematic representation of the genomic structure of the mouse SGLT2 promoter. (**B**) Bandshift experiments on the SGLT2 promoter HNF1 binding sites. A β-fibrinogen HNF1 binding site is shown as control. (**C**) HNF1α-dependent transactivation of SGLT2 promoter constructs in C33-transfected cells. Either long (2 kb) or short (400 bp) promoter fragments driving a luciferase reporter cassette were cotransfected together with 1 μg of HNF1α expression vector (RSV–HNF1) (Chouard *et al.*, 1990).

suggests that the mRNA decrease induces a reduction of the corresponding protein. Altogether, our data clearly demonstrate that the physiological contribution of SGLT2 to the renal glucose reabsorption is crucial and cannot be completely compensated for by the residual SGLT1 and SAAT1 activities.

Previously we have shown that HNF1α-deficient animals suffer from a severe insulin secretion defect. However, mutant animals do not develop a particularly elevated hyperglycemia (Pontoglio *et al.*, 1998). This could be explained by the fact that in *HNF1α*$^{-/-}$ animals the insulin secretion defect is partially compensated for by the renal glucose reabsorption defect. The cloning of the SGLT2 murine gene allowed the identification of HNF1α binding sites in the transcriptional control region of the gene. The HNF1α-dependent expression of SGLT2 was further corroborated by cotransfection experiments.

In the past there has been a long debate as to whether patients with renal glucosuria are predisposed to develop diabetes mellitus with time. It was reported that 63% of patients initially suffering from renal glucosuria developed overt diabetes in a period of 3 months to 13 years (Ackerman *et al.*, 1958). More

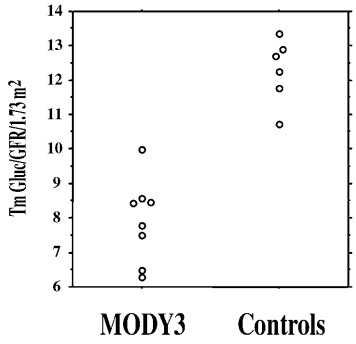

Fig. 4. Maximal glucose transport in MODY3 patients. MODY3 maximal glucose transport values (Tm/GFR/1.73 m^2 expressed in mM/1.73 m^2), calculated as described in Methods, are plotted in comparison with the values obtained with type-2-diabetic non-MODY3 subjects.

recently, an excessive glucosuria linked to a decreased renal threshold for glucose was reported in diabetic patients from MODY families (Tattersall, 1974; Menzel *et al.*, 1998). Our results clearly confirm that all MODY3 subjects belonging to unrelated families exhibit the renal defect. The fact that diabetes mellitus and low renal glucose threshold coexist in MODY3 carriers lends further support to the idea that renal glucosuria with normal serum glucose concentration could be considered a predictive sign of MODY3 diabetes.

METHODS

Animals. All studies were conducted on animals of 2–3 weeks of age. *HNF1α* mutant homozygotes, heterozygotes and wild-type animals were age matched. The genetic background of the animals was either C57/Bl6 X 129sv or 129sv/DBA2/C57/bl6.

Glucose and alanine uptake. Uptakes were performed on suspension of renal proximal tubules prepared as described elsewhere (Essig *et al.*, 1998). Briefly, for each experiment, tubules from four animals were used. Tubules were resuspended in a medium containing either sodium or *N*-methyl-glucamine and uptakes were performed as previously described (Friedlander and Amiel, 1989).

RNA analysis. Total RNA was isolated as described elsewhere (Chomczynski and Sacchi, 1987). For northern blots, 15 µg of total RNA were separated in 0.66 M formaldehyde, 1% agarose gels and transferred to Hybond N (Amersham) nylon membranes. Single-stranded DNA probes were labelled either by linear PCR as described elsewhere (Pontoglio *et al.*, 1997) or by random priming. HNF1α probe corresponded to HNF1α C-terminal domain from position *Acc*I (890) and *Bam*HI (2354) of the plasmid RSV–HNF1 (Chouard *et al.*, 1990). SGLT1 probe was produced by RT–PCR on renal RNA with the following oligonucleotides: 5′-GCCCATCCCAGACGTACAC-3′ (plus strand) and 5′-GGTCCAGCCCACAGAACAG-3′ (minus strand). These two oligonucleotides amplify a fragment of 178 nt on random hexamer primed cDNA. SGLT2 probe was produced with RT–PCR using oligonucleotides at position 6–29 and from the complementary sequence at position 134–157 of the RNU29881 sequence (You *et al.*, 1995). SAAT1 probe was obtained by PCR on murine genomic DNA with primers corresponding to position 130935–130956 and 131314–131293 of the ac005818 sequence that was found to contain the murine SAAT1 gene. D2/NAA-Tr was obtained by PCR using the oligonucleotides at position 65–89 and from the complementary sequence at position 565–541 of the D2/NAA-Tr sequence DDBJ/EMBLGenBank accession No. M80804.

Table I. Demographic, genetic and clinical profile of MODY3 and control subjects

Subject	Kindred	Mutation	Sex	Age (years)	BMI (kg/m^2)	Glucose tolerance status[a]	Age of diagnosis (years)	Fasting glucose (mM)	Treatment	Glucose Tm mM/1.73m^2
M3#1	F593	R55G56fsdelGAGGG	M	41	24.6	DM	17	7.0	Sulf.	7.8
M3#2	F593	R55G56fsdelGAGGG	F	45	21.6	DM	18	7.4	Sulf.	7.5
M3#3	F515	S142F	M	42	21.3	NGT	–	5.7	–	6.29
M3#4	F515	S142F	F	29	27.2	DM	9	8.1	Sulf. + Biguan.	8.5
M3#5	F703	R171X	F	21	20.9	DM	14	11.1	Sulf. + Biguan.	10.1
M3#6	F703	R171X	F	48	25.2	DM	17	5.3	Sulf. + Biguan.	6.5
M3#7	F706	IVS5nt + 2T→A	M	42	24.1	DM	20	5.6	Biguan.	8.6
M3#8	F706	IVS5nt + 2T→A	M	39	19.3	DM	20	6.4	Biguan.	8.4
MODY3 subjects (m ± SD)			4M/4F	38.4 ± 3.2	23.0 ± 0.9		16.4 ± 3.9	7.1 ± 1.9		7.9 ± 0.4
Control group n = 6 (m ± SD)			1M/5F	41.3 ± 4.4	26.7 ± 2.6	DM	36.3 ± 5.1	10.6 ± 3.9	see below[b]	12.3 ± 0.4
Statistical significance				ns	ns					p <0.0001

Glucose and phosphate maximal tubular reabsorption capacity in MODY3 patients. Glucose Tm stands for glucose maximal transport capacity and is expressed in mM/1.73m^2. The mean glomerular filtration rate was similar in MODY3 and control subjects (105.4 ± 6.6 vs 106.8 ± 12.2). Confidence intervals (95%) for Glucose Tm (McPhaul and Simonaitis, 1968) and for the other parameter are indicated below. Family and patient numbers are indicated (Vaxillaire *et al.*, 1997).
[a]NGT and DM stand for normal glucose tolerance and diabetes mellitus, respectively. 'ns', non-significant; 'Sulf.', Sulfonylurea; 'Biguan.', Biguanides.
[b]The treatment for the controls was: Sulfonylurea + Biguanides (two patients); Insulin (two patients); Insulin + Biguanides (two patients).

GLUT2 probe was derived with *Eco*RI digestion of the plasmid prGLUT2 kindly provided by Dr G.I. Bell. Na^+/K^+ ATPase α probe was kindly provided by Dr N. Farman.

Screening of a mouse genomic library and isolation of SGLT2 regulatory regions. The first 500 bp of the rat SGLT2 cDNA (AN U29881) was labelled by random priming and used to screen a λDASH mouse genomic library. Approximately 7×10^5 plaques were lifted onto nylon filters (Hybond N, Amersham) and hybridized with the SGLT2 probe as recommended by the manufacturer. Positive clones were isolated and subcloned into pUC20 (Promega).

Nuclear extracts and bandshift experiments. Nuclear extracts from freshly dissected liver were performed as previously described (Cereghini *et al.*, 1987) except that ammonium sulfate precipitation and dialysis were omitted. Protein concentrations were determined by the Bradford method. For bandshift assays, double-stranded oligonucleotides were labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Labelled probes (1 ng) were incubated with 1 µg of a bacterially expressed N-terminal HNF1α moiety (281 amino acids) (HNT) or 10 µg of liver nuclear extracts, for 10 min in a final volume of 14 µl as described previously (Cereghini *et al.*, 1988) and run on a 5% polyacrylamide gel in 0.25× TBE.

Cell line, transient transfection and luciferase assay. The C33 human epithelial cervical carcinoma cells (Yee *et al.*, 1985), which lack endogeneous HNF1α expression, were cultured and transfected as previously described (Bach and Yaniv, 1993). For each transfection, a total of 5 µg of DNA was used including 2 µg of reporter plasmids (2kb-luci or 400bp-luci), 1 µg of HNF1α expression vectors and 1 µg of pRSV-β-galactosidase construct for normalization of transfection efficiencies. The total quantity of plasmids was maintained constant by adding suitable amounts of a pGEM3 plasmid. Forty to forty-eight hours after transfection, the cells were harvested and total cell extracts were prepared. β-galactosidase activities were measured as previously described (Gorman *et al.*, 1982) and luciferase assays were performed as recommended by the manufacturer (Promega). Each experiment was repeated 3–5 times with at least two different plasmid preparations.

Patients. Renal function was assessed in eight MODY3 patients carrying mutations in the HNF1α gene at the heterozygous state. Patients belonged to four different MODY3 kindreds with different HNF1α mutations (Vaxillaire *et al.*, 1997; Chevre *et al.*, 1998). Subjects with HNF1α mutations had normal glucose tolerance (NGT; $n = 1$) or diabetes mellitus (DM; $n = 7$) according to WHO criteria. All subjects were in good general health; those with diabetes had no evidence of diabetic complications. Six unrelated individuals with type 2 diabetes mellitus were used as controls for these evaluations (Table I). Glomerular filtration rates were within normal range. In the four male patients with HNF1α mutations (M3#1, M3#3, M3#7, M3#8), glucosuria was discovered during military service. Diabetes was diagnosed in three of them. Patient M3#3, although exhibiting glucosuria, is still non-diabetic according to the WHO criteria.

Determination of theoretical threshold for renal glucose excretion (Tm/GFR). Glucose titration curves were determined according to a standard method (McPhaul and Simonaitis, 1968; Haycock, 1998). Briefly, urinary glucose excretion was measured in each subject exposed at different glucose plasma levels. Serum glucose concentrations (abscissae) were plotted against urinary glucose excretions (*y*-axis). The intercept of the linear regression curve with the *x*-axis was considered as the Tm gluc/GFR (Haycock, 1998).

Renal investigations were performed in the morning in fasting subjects. Glomerular filtration rate was assessed by polyfructosan clearance. Urinary samples were collected by spontaneous voiding. Urinary flow was maintained >4 ml/min throughout the investigation by oral water loading (10 ml/kg body weight initially, then 150 ml every 30 min). Serum and urinary concentrations of polyfructosan were determined every 30 min for 3 h. To determine maximal renal reabsorption capacity for glucose (Tm gluc), patients were injected with 30% solution of glucose (1 ml/kg body weight). Serum glucose concentrations were determined before and 3, 10, 20, 30, 60 and 90 min after glucose loading. Urinary glucose excretion were measured at 10, 30, 60 and 90 min after loading.

Statistical analysis. Results are expressed as mean ± SE. Statistical significance was evaluated using the Student's *t*-test.

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